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Supplemental Assay Method for the Titration of Bovine Rhinotracheitis Neutralizing Antibody (Constant Virus-Varying Serum Method)

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1. Introduction

This Supplemental Assay Method (SAM) describes an *in vitro* test of serum for the serum neutralization (SN) antibody titer against bovine rhinotracheitis virus (IBR). The assay uses a constant amount of virus to test varying dilutions of serum in a cell culture system. The assay meets the requirements in the Code of Federal Regulations, Title 9 (9 CFR) 113.216 to test serum samples for potency testing of inactivated IBR vaccines.

NOTE: For this SAM, the dilution terminology of 1:2, 1:4, etc. specifies 1 part + 1 part (liquid), 1 part + 3 parts, etc.

2. Materials

2.1 Equipment/instrumentation

Equivalent equipment or instrumentation may be substituted for any brand name listed below.

2.1.1 Incubator, 36° ± 2°C, 5% ± 1% CO_2, high humidity (Model 3158, Forma Scientific, Inc.)

2.1.2 Vortex mixer (Vortex-2 Genie, Model G-560, Scientific Industries, Inc.)

2.1.3 Microscope, inverted light (Model CK, Olympus America, Inc.)

2.1.4 Micropipettor, 200-µl, 500-µl, 1000-µl and tips

2.1.5 Water bath

2.2 Reagents/supplies

Equivalent reagents or supplies may be substituted for any brand name listed below. All reagents and supplies must be sterile.

2.2.1 IBR Reference virus, Cooper strain [available from the Center for Veterinary Biologics (CVB)]

2.2.2 Madin-Darby bovine kidney (MDBK) cells

2.2.3 Minimum essential medium (MEM)

2.2.3.1 9.61 g MEM with Earles salts without bicarbonate

2.2.3.2 2.2 g sodium bicarbonate ($NaHCO_3$)

2.2.3.3 Dissolve **Sections 2.2.3.1 and 2.2.3.2** with 900 ml deionized water (DW).

2.2.3.4 Add 5 g lactalbumin hydrolysate or edamine to 10 ml DW. Heat to 60° ± 2°C until dissolved. Add to **Section 2.2.3.3** with constant stirring.

2.2.3.5 Q.S. to 1000 ml with DW; adjust pH to 6.8-6.9 with 2N hydrochloric acid (HCl).

2.2.3.6 Sterilize through a 0.22-µm filter.

2.2.3.7 Aseptically add:

1. 10 ml L-glutamine

2. 50 µg/ml gentamicin sulfate

2.2.3.8 Store at 4° ± 2°C.

2.2.4 Growth Medium

2.2.4.1 900 ml of MEM

2.2.4.2 Aseptically add 100 ml gamma-irradiated fetal bovine serum (FBS)

2.2.4.3 Store at 4° ± 2°C.

2.2.5 Maintenance Medium

2.2.5.1 980 ml of MEM

2.2.5.2 Aseptically add 20 ml of gamma-irradiated FBS

2.2.5.3 Store at 4° ± 2°C.

2.2.6 Positive IBR control serum (PCS) [available from CVB]

2.2.7 Negative IBR control serum (NCS)

2.2.8 Cell culture plates, 96-well

2.2.9 Polystyrene tubes, 12 x 75-mm

3. Preparation for the test

3.1 Personnel qualifications/training

Personnel must have training in antibody titration assays, cell culture maintenance, and in the principles of aseptic techniques.

3.2 Preparation of equipment/instrumentation

3.2.1 On the day of test initiation, set a water bath at 36° ± 2°C.

3.2.2 On the day of test initiation, set a water bath at 56° ± 2°C.

3.3 Preparation of reagents/control procedures

3.3.1 Two days prior to test performance, seed 96-well cell culture plates with MDBK cells, in Growth Medium, at a cell count ($10^{4.7}$ to $10^{5.2}$ cells/ml) that will produce a confluent monolayer after 48 ± 8 hours of incubation at 36° ± 2°C in a CO_2 incubator. These become the MDBK Plates. Four Test Sera may be tested per MDBK Plate.

3.3.2 On day of test initiation:

3.3.2.1 Working IBR Reference. Rapidly thaw a vial of IBR Reference in a 36° ± 2°C water bath. Dilute the virus in MEM to contain 50-300 50% tissue culture infective dose ($TCID_{50}$)/25 µl.

3.3.2.2 Virus Back Titration. Make 3 serial tenfold dilutions of Indicator Virus.

1. Place 900 µl of MEM into 3, 12 x 75-mm polystyrene tubes labeled 10^{-1} x 10^{-3}.

2. Transfer 100 µl of Working IBR Reference to the 10^{-1} tube; mix by vortexing. Discard pipette tip.

3. Using a new pipette tip, transfer 100 µl from the 10^{-1} tube to the 10^{-2} tube; mix by vortexing.

4. Using a new pipette tip, transfer 100 µl from the 10^{-2} tube to the 10^{-3} tube; mix by vortexing.

3.4 Preparation of the Test Serum, PCS, and NCS

3.4.1 On the day of test initiation, heat inactivate all Test Serum samples, PCS, and NCS in a 56° ± 2°C water bath for 30 ± 5 minutes.

3.4.2 Prepare serial twofold dilutions of the Test Serum samples, PCS, and NCS in a 96-well cell culture plate, which becomes the Dilution Plate (**Appendix I**). A sufficient number of dilutions of the PCS should be performed to reach an endpoint for the control. Twofold dilutions are made as follows:

1. Add 150 µl MEM to all wells in Rows B through D. Rows E through H may be used if additional samples are to be tested.

2. Add 150 µl Test Serum Samples, PCS, or NCS to Rows A and B. Mix Row B with the multichannel micropipettor (7 ± 2 fills by aspiration and expulsion of the 12-channel micropipettor).

3. Using new pipette tips, transfer 150 µl from Row B to Row C. Mix Row C with the multichannel micropippetor (7 ± 2 fills).

4. Repeat as in **Section 3.4.2(3)** for Row D, and discard 150 µl from all wells in Row D. Additional dilutions of Test Serum samples may be prepared if endpoint titers are desired.

5. Add 150 µl of Working IBR Reference virus to all wells of the Dilution Plate; tap plates gently to mix. Incubate for 60 ± 10 minutes at 36° ± 2°C to allow for neutralization of virus. The addition of virus results in an additional twofold dilution of Test Serum.

4. Performance of the test

4.1 On the day of test initiation, decant Growth Medium from the MDBK Plates.

4.2 Inoculate 50 µl/well of each Virus-Serum mixture (**Section 3.4.2[5]**) into 5 wells/dilution of a MDBK Plate (**Appendix II**).

4.3 Inoculate 25 µl of each dilution of Virus Back Titration into 5 wells of a MDBK Plate.

4.4 Add 25 ul of MEM to each Virus Back Titration well

4.5 Maintain 5 or more wells on a MDBK Plate as uninoculated cell controls.

4.6 Incubate MDBK Plates for 60 ± 10 minutes at 36° ± 2°C in a CO_2 incubator.

4.7 Add 200 µl/well of Maintenance Medium to all wells (do not remove Virus-Serum mix). Incubate MDBK Plate for 4 days ± 12 hours postinoculation at 36° ± 2°C in a CO_2 incubator.

4.8 At 4 days postinoculation, examine the wells with an inverted light microscope at 100X magnification. The CPE of IBR is visible as grape-like clusters of rounded cells in the cell monolayer where the cells have been destroyed by the virus.

4.8.1 Record the number of wells/dilution showing any characteristic CPE of IBR for each Test Serum and Virus Back Titration.

4.9 Calculate the titer of the Test Serum, PCS, and NCS using the Spearman-Kärber method as commonly modified.

Example:

1:2 dilution of Test Serum = 5/5 wells CPE negative
1:4 dilution of Test Serum = 5/5 wells CPE negative
1:8 dilution of Test Serum = 3/5 wells CPE negative
1:16 dilution of Test Serum = 0/5 wells CPE negative

Spearman-Kärber formula:

Test Serum titer = (X – d/2 + [d • S]) where:

X = $\log_{10}$ of lowest dilution (= 0.3)
d = $\log_{10}$ of dilution factor (= 0.3)
S = sum of proportion of CPE negative

$$\frac{5}{5} + \frac{5}{5} + \frac{3}{5} + \frac{0}{5} = \frac{13}{5} = 2.6$$

Test Serum titer = (0.3 – 0.3/2 + [0.3 • 2.6) = 0.93
antilog of 0.93 = 8.5

From the example, the Test Serum has a SN titer of 1:8.5.

4.10 By the same Spearman-Kärber method, calculate the endpoint of the BVDV Back Titration. The titer is expressed as $\log_{10}$ tissue culture infective doses 50 ($TCID_{50}$) per 25 µl dose.

Example:

10^{0} dilution of the Working BVDV = 5 of 5 wells CPE Positive
10^{-1} dilution of the Working BVDV = 5 of 5 wells CPE Positive
10^{-2} dilution of the Working BVDV = 3 of 5 wells CPE Positive
10^{-3} dilution of the Working BVDV = 0 of 5 wells CPE Positive

Test Serum titer = (X – d/2 + [d • S]) where:

X = $\log_{10}$ of lowest dilution (= 0)
d = $\log_{10}$ of dilution factor (= 1)
S = sum of proportion of CPE positive

$$\frac{5}{5} + \frac{5}{5} + \frac{3}{5} + \frac{0}{5} = \frac{13}{5} = 2.6$$

Test Serum titer = (0 – 1/2 + [1 • 2.6) = 2.1
antilog of 2.1 = 125.9

Titer of the Working BVDV is 126 $TCID_{50}$/25 µl dose in the test.

5. Interpretation of the test results

5.1 Validity requirements:

5.1.1 No visible contamination or serum toxicity should be observed in more than one well/dilution of any dilution of a Test Sera or the FBS.

5.1.2 The SN titer of the PCS should vary by no more than twofold from its mean titer as established from a minimum of 10 titrations that have been previously performed.

5.1.3 The SN titer of the NCS should be <1:2.

5.1.4 The Virus Back Titration must have between 50-300 $TCID_{50}$/25 µl.

5.2 Four out of 5 postvaccinated Test Sera shall have a SN titer ≥1:8 for a **SATISFACTORY** result as stated in 9 CFR 113.216.

5.3 Retest

5.3.1 If the initial test is valid and less than 4 out of 5 postvacinnated Test Sera have a SN titer <1:8, the test is repeated (1st retest).

5.3.2 If the 2nd valid test (1st retest) confirms the initial results, the Test Serial is **UNSATISFACTORY**.

5.3.3 If the 2nd valid test (1st retest) fails to confirm the initial result, the Test Sera is tested a 3rd time (2nd retest).

5.3.3.1 If 4 out of 5 postvaccinated Test Sera have a SN titer ≥1:8 in the 2nd and 3rd valid tests (1st and 2nd retests), the Test Serial is **SATISFACTORY**.

5.3.3.2 If the 3rd valid test (2nd retest) confirms the initial result, the Test Serial is **UNSATISFACTORY**.

6. Report of test results

Record the SN titers on the test record.

7. References

7.1 Code of Federal Regulations, Title 9, Part 113.216, U.S. Government Printing Office, Washington, DC, 2005.

7.2 Finney, DJ. *Statistical Method in Biological Assay*. 3rd ed. Charles Griffin and Company, London, 1978.

7.3 Rose NR, H Friedman, JL Fahey, eds. *Manual of Clinical Laboratory Immunology*. Chapter 11: Neutralization Assays. ASM, Washington, DC, 1986.

8. Summary of revisions

This document was revised to clarify practices currently in use at the Center for Veterinary Biologics and to provide additional detail.

The assay was converted from a virus plaque method utilizing Bovine Embryonic Kidney cell cultures to a microtiter cytopathic effect assay utilizing Madin Darby Kidney cell cultures.

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9. Appendices

Appendix I
Transfer Plate

A 1:2*	TS1	TS2	TS3	TS4	TS5	TS6	TS7	TS8	TS9	TS 10	PSC	NSC
B 1:4	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
C 1:8	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
D 1:16	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
E 1:2*	TS11	TS12	TS13	TS14	TS15	TS16	TS17	TS18	TS19	TS20	↓	↓
F 1:4	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
G 1:8	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
H 1:16	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓

***Final serum dilutions**
TS= Test Serum (diluted 1:2 - 1:16)
PCS= Positive control serum (final dilutions 1:2-1:16)
NCS= Negative control serum (final dilutions 1:2-1:16)

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Appendix II
Test Plate

A 1:2	TS1	TS1	TS1	TS1	TS1	CC	CC	TS2	TS2	TS2	TS2	TS2
B 1:4												
C 1:8												
D1:16												
E 1:2	TS3	TS3	TS3	TS3	TS3	CC	CC	TS4	TS4	TS4	TS4	TS4
F 1:4												
G 1:8												
H 1:16												

TS= Test Serum CC= Cell Control